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Sex differences and estrous cycle effects on foreground contextual fear conditioning

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HIGHLIGHTS

- Male and female rats were tested in paired and explicitly unpaired fear conditioning.
- Sex hormones influenced primarily unpaired conditioned responses in females.
- BDNF protein levels differed by sex but not by fear conditioning procedure.

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ABSTRACT

Previous reports have suggested that the female estrous cycle modulates fear responses to contextual and discrete cue stimuli following fear conditioning, but conflicting results have been obtained across studies. We assessed sex differences between males and naturally cycling female rats using fear conditioning procedures that involve learning about a discrete cue (tone conditioned stimulus, CS) and either background (explicitly paired) or foreground (explicitly unpaired) contextual conditioning. We measured brain derived neurotrophic factor (BDNF) expression in amygdala, hippocampus, and medial prefrontal cortex, brain regions implicated in the fear circuitry. When comparing males to females without considering the estrous cycle phase, sex differences were found in unpaired conditioning only; females showed significantly less freezing than males during acquisition. Significant differences in unpaired but not paired conditioning were found between subgroups of females depending on the phase of the estrous cycle. Female rats that underwent unpaired conditioning in estrus showed significantly less freezing during acquisition and cued recall than males. In contrast, contextual recall in females that received unpaired training was not affected by estrous cycle phase. BDNF protein expression remained unchanged by fear conditioning but overall males expressed higher levels in all brain regions examined compared to females. Together, these results show that sex differences in fear behaviors following foreground contextual conditioning depend on the phase of the estrous cycle. These results are discussed in light of previous reports of sex and estrous cycle effects on fear learning and BDNF.

1. Introduction

It is widely accepted that different forms of fear memory rely on distinct neurobiological events during consolidation. The amygdala, hippocampus and prefrontal cortex are elements of the neural circuitry underlying associative fear memory formation [1,2] and synaptic plasticity within this circuitry is required for the formation, consolidation and expression of fear-associated behaviors [3,4]. For instance, memory consolidation of fear learning requires protein synthesis in the amygdala [5], prefrontal cortex [6] and hippocampus [7,8], and numerous studies have confirmed that the prefrontal cortex-amygdala circuits are essential for fear extinction learning [6,9]. Moreover, there is considerable evidence that the hippocampus has an essential role in contextual fear conditioning [2,10,11]. Interestingly, memory formation and consolidation is sexually dimorphic and appears to be modulated by hormone-dependent mechanisms of associative fear circuitry [12–17]. In the hippocampus, high levels of estrogen correlate with increased density of synapses [18]. Similarly, in the prefrontal cortex, estrogen administration increases dendritic spine density and AMPA mediated glutamatergic synapses [19]. These effects of estrogen on synaptic density and efficacy are believed to underlie learning differences in females.

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Several studies have reported that female rats exhibit less freezing during contextual and cued fear conditioning compared to males [13,15,16], although conflicting results have been reported. For instance, in one study females in proestrus during training and testing, a phase characterized by high levels of estrogen and progesterone, differed from males in contextual fear but not cued fear retention [20]. Milad et al. [21] reported no difference between males and females in fear conditioning, extinction and recall, but when females were divided into low and high estrogen levels (metestrus and proestrus, respectively), females that underwent extinction during the metestrus phase showed more fear during extinction recall than males [21]. These findings propose that estrogen (and/or sex hormones) may regulate plasticity that underlies associative fear memory.

Further evidence for a role of sex hormones in modulating fear associative learning comes from studies on the activity-regulated neurotrophic factor, brain derived neurotrophic factor (BDNF). BDNF plays essential roles in mediating long and short term plasticity associated to fear learning [22,23]. BDNF mRNA is induced by contextual fear conditioning in the CA1 region of the hippocampus [24], in the basolateral amygdala [25] and, following contextual fear extinction, in the medial PFC (mPFC) [26]. BDNF supports hippocampal and amygdalar long-term potentiation (LTP) [27,28] and modulates memory consolidation of conditioned fear in amygdala and hippocampus [24,29,30] or extinction of conditioned fear within the PFC [31,32]. Importantly, BDNF levels are regulated by the estrous cycle [33–36]. Gibbs [33] reported higher levels of BDNF mRNA in CA1 and CA3/4 areas of the hippocampus during early diestrus and lower levels of BDNF during late proestrus. In the same study, ovariectomized rats exhibited increases in BDNF mRNA and decreases in BDNF protein after estrogen treatment. Other work has shown that hippocampal BDNF protein levels are increased during estrus and proestrus relative to metestrus or ovariectomized rats and that these BDNF protein cycle-dependent differences correlate with electrophysiological responses in CA1 and CA3 [34]. In another study, BDNF mRNA levels were lower in the dentate gyrus granule cell layer and in the mPFC during proestrus [35]. Although these studies describe contradictory findings they suggest that interactions between estrogen and BDNF signaling may underlie sex differences in fear associative learning by mediating plasticity within the amygdala-hippocampus-prefrontal cortex circuitry.

Taking into account the evidence, estrogen has been linked to learning-related synaptic plasticity and regulation of BDNF, and both estrogen and BDNF are linked to behavioral responses to emotionally salient stimuli [37,38].Therefore, we hypothesized that fear conditioning would differ between males and females and as a function of estrous cycle. We further hypothesized that fear conditioning and estrous cycle regulates BDNF protein levels. To this end we employed two learning procedures – explicitly paired and explicitly unpaired fear conditioning – to examine sex differences in fear acquisition and retention and in BDNF expression following learned associations that involve distinct cue and contextual processing. In paired fear conditioning, a discrete association is formed between the conditioned tone stimulus (CS) and the unconditioned stimulus (US). Under these conditions, the CS becomes the main predictor of the shock. In contrast, in unpaired conditioning, the CS and US are explicitly unpaired, and the tone acquires less predictive value than in paired conditioning [1,39,40]. In both paired and unpaired conditioning fear responses emerge to the context, but research suggests that each involves distinct contextual processing (background vs. foreground contextual conditioning, respectively) and underlying mechanisms [1,41–46].We analyzed freezing behavior during cued and contextual recall following paired and unpaired fear conditioning as a function of sex and estrous cycle in female rats. In addition, we explored if paired and unpaired fear conditioning regulate BDNF protein levels in amygdala, prefrontal cortex and hippocampus of males and females.

2. Materials and methods

2.1. Subjects

Thirty-six adult male and 105 female Wistar rats (obtained from Harlan, Mexico and bred in INDICASAT colonies) were used for this study. Rats were housed in groups of four to five and maintained at 22 ± 1 °C on a 12 h light/dark cycle with free access to food and water. Experiments were conducted during the dark cycle. All experiments were conducted in accordance with the National Institute of Health regulations regarding the care and use of laboratory animals and INDICASAT policies.

2.2. Apparatus

Male and female rats were handled three days prior to the experiments. Experiments were performed as described previously [47,48]. Briefly, rats were placed in a conditioning chamber (27 cm × 28 cm × 30 cm) contained within an isolated sound- and light-attenuating chamber. The chamber floor consisted of stainless steel bars capable of delivering a mild electric shock. A computer software program (Graphic State, Coulbourn Instruments) was used to generate and deliver stimulus protocols. The conditioned stimulus (CS), a 30 s tone with an intensity of 90 dB, was produced through speakers mounted on the chamber walls. The unconditioned stimulus (US) was a 1 s footshock with an intensity of 0.8 mA. A low-light camera placed inside the chamber recorded all conditioning sessions for offline scoring of behavior.

2.3. Behavioral procedures

Vaginal lavage samples were collected from females daily beginning 10 days prior to the experiments to confirm normal cycling and on days in which experiments took place [21]. The vaginal epithelial cells were examined under a light microscope and the cycle phase was categorized according to Becker et al. [49]. Only regularly cycling females were included in the study. Samples were taken between 12:30 and 1:30 P.M. and rats were returned to their cages for 60 min before the experiments. To assess the influence of the estrous cycle on acquisition of fear learning, all females underwent acquisition experiments on different phases of the estrous cycle: estrus, metestrus, diestrus and proestrus.

Animals were assigned to one of three groups in the following manner: paired conditioning, unpaired conditioning or tone-alone. During paired conditioning, rats were placed into the conditioning chamber and received five tones that co-terminated with a footshock. The inter-trial interval (ITI) for the tone-shock pairing was 5 min. The unpaired conditioning protocol consisted of five explicitly unpaired presentations of the CS and US, where the shock-to-tone interval was 120 s and the tone-to-shock interval was 180 s. The tone-alone control group received five presentations of the CS with no shock, and the CS was delivered at the same point in time as in the paired conditioning protocol. Each training procedure lasted 28 min. Cued recall and contextual recall were tested 24 and 48 h after conditioning in novel and training contexts, respectively. In the novel context, rats were exposed to three tone presentations (ITI = 180 s) in a modified conditioning chamber. In the training context, freezing responses to three tone presentations (ITI = 180 s) were measured 48 h after conditioning. At the conclusion of each session, animals were returned to their home cage. Freezing was used as the index of behavioral fear [50] and was defined as a complete absence of movement except that required for breathing [51].

2.4. BDNF assays

A group of 30 females and 15 male rats were housed in the same conditions as described in section 1.1. Males and females underwent